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Heteroresistance to the model antimicrobial peptide polymyxin B in the emerging *Neisseria meningitidis* linage11.*2* urethritis clade: mutations in the *pilMNOPQ* operon

Running title: Heteroresistance to polymyxin in *Neisseria meningitidis*

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Key words: *Neisseria meningitidis*, meningococcal urethritis, heteroresistance, polymyxin B, antimicrobial peptide, pilQ

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Heteroresistance to the model antimicrobial peptide polymyxin B in the emerging *Neisseria meningitidis* linage11.*2* urethritis clade: mutations in the *pilMNOPQ* operon

Summary

Clusters of *Neisseria meningitidis* (Nm) urethritis among primarily heterosexual males in multiple United States cities have been attributed to a unique non-encapsulated meningococcal clade (the U.S. Nm urethritis clade, US_NmUC) within the hypervirulent clonal complex 11. Resistance to antimicrobial peptides (AMPs) is a key feature of urogenital pathogenesis of the closely related species, *N. gonorrhoeae*. The US_NmUC isolates were found to be highly resistant to the model AMP, polymyxin B (PmB, MICs 64-256 μg/ml). The isolates also demonstrated stable subpopulations of heteroresistant colonies that showed near total resistant to PmB (MICs 384-1024 μg/ml) and colistin (MIC 256 μg/ml) as well as enhanced LL-37 resistance. This is the first observation of heteroresistance in *N. meningitidis*. Consistent with previous findings, overall PmB resistance in US_NmUC isolates was due to active Mtr efflux and LptA-mediated lipid A modification. However, whole genome sequencing, variant analyses and directed mutagenesis revealed that the heteroresistance phenotypes and very high level AMP resistance were the result of point mutations and IS1655 element movement in the *pilMNOPQ* operon, encoding the type IV pilin biogenesis apparatus. Cross-resistance to other classes of antibiotics was also observed

in the heteroresistant colonies. High-level resistance to AMPs may contribute to the pathogenesis of US_NmUC.

Key words: *Neisseria meningitidis*, meningococcal urethritis, heteroresistance, polymyxin B, antimicrobial peptide, pilQ

Introduction

Neisseria meningitidis (Nm), an obligate human pathogen, is carried asymptomatically in the nasopharynx of 5-10% of adults and is transmitted by close contact with respiratory droplets of oral or nasal secretions. Nm is also a leading cause of meningitis and rapidly fatal sepsis in otherwise healthy individuals that can cause large epidemic outbreaks (Rouphael & Stephens, 2012, Stephens *et al.*, 2007). While capsular polysaccharide conjugate and protein-based meningococcal vaccines provide protection, invasive meningococcal disease is a continued worldwide problem.

Nm and *Neisseria gonorrhoeae* (Ng) are the only two *Neisseria* species that are human pathogens and these organisms most commonly colonize respiratory and urogenital tracts, respectively. Historically, Nm has not been a significant cause of urogenital disease and was infrequently recovered from the urogenital tract (cervix, vagina, and urethra) and rectum. However, there have been sporadic case reports in which Nm was isolated from patients with urethritis, cervicitis, vaginitis, proctitis, pelvic inflammatory disease, and postpartum endometritis dating back to the 1940s (Givan *et al.*, 1977, Conde-Glez & Calderon, 1991, Maini *et al.*, 1992). Recently, sustained sexually transmitted meningococcal urethritis outbreaks have been reported (Bazan *et al.*, 2016, Bazan *et al.*, 2017, Toh *et al.*, 2017, Tzeng *et al.*, 2017, Retchless *et al.*, 2018). In one study, seventy-five Nm urethritis cases detected between January and November of 2015 in Columbus OH, represented 20%

of all men who presented during that time with urethral GNID and growth of oxidase-positive Gram-negative diplococci (Bazan *et al.*, 2017). Similar Nm-associated urethritis clusters have now been observed in multiple U.S. cities (Bazan *et al.*, 2016, Bazan *et al.*, 2017, Toh *et al.*, 2017, Retchless *et al.*, 2018). These urethritis-associated Nm isolates are members of a novel nongroupable US Nm urethritis clade (US_NmUC) in the cc11/ET-15 hyperinvasive lineage (Tzeng *et al.*, 2017). The US_NmUC isolates do not make capsules (Toh *et al.*, 2017, Tzeng *et al.*, 2017) due to an IS1301 insertion that caused a multi-gene deletion at the capsule biosynthesis locus (Tzeng *et al.*, 2017). Unlike many Nm isolates, the US_NmUC isolates are capable of efficient nitrite dependent anaerobic growth. This is due to a gene conversion event that introduced gonococcal *aniA-norB* genes, which encode enzymes that catalyze conversion of nitrite to nitric oxide and then nitrous oxide (Tzeng *et al.*, 2017). Thus, the emergence of US_NmUC as a urethritis pathogen is likely the result of multiple evolutionary genetic events that allow better assimilation into the same niche first adopted by gonococci (Tzeng *et al.*, 2017, Retchless *et al.*, 2018).

Resistance to host-derived antimicrobial peptides (AMPs) is a key feature of neisserial pathogenesis at mucosal surfaces (Johnson & Criss, 2011, Tzeng & Stephens, 2015). A hallmark of gonococcal (and meningococcal) urethritis is the influx of PMNs, which employ both oxidative (production of reactive oxygen species) and non-oxidative (release of AMPs) killing mechanisms (Johnson & Criss, 2011, Criss & Seifert, 2012). In both Nm and Ng, resistance to PMN- derived and epithelial derived AMPs is mainly due to the activity of the LptA-mediated lipid A modification by phosphoethanolamine and the Mtr efflux pump (Tzeng *et al.*, 2005). We have previously performed *mariner* random mutagenesis screening and identified transposon mutants in *pilM* and *pilP* that increased AMP resistance (Tzeng *et al.*, 2005). In this report we show that the US_NmUC isolates are highly resistant to the AMP

polymyxin B (PmB), a well-recognized surrogate for endogenous AMPs, and also exhibit "heteroresistance" (subpopulations of higher resistant colonies in the zone of inhibition) to PmB and colistin (polymyxin E). Increased AMP resistance of these isolates is linked to mutations in *pilM* and *pilQ*. PilQ is a member of the secretin family of proteins, and a major component of the outer membrane (Berry *et al.*, 2012). PilM is a cytoplasmic ATP-binding protein that together with PilN/O/P proteins forms the inner membrane platform of the type IV pilus biogenesis complex (Ayers *et al.*, 2009). Nm heteroresistance selected by PmB exposure, in addition to enhance resistance to colistin and LL-37, also conferred cross-resistance to several antibiotics, suggesting entry of these antibiotics is PilQ dependent. Heteroresistance to PmB has been described in several other Gram-negative bacterial pathogens, (Li *et al.*, 2006, Lo-Ten-Foe *et al.*, 2007, Hermes *et al.*, 2013, Hjort *et al.*, 2016, Jayol *et al.*, 2015, El-Halfawy & Valvano, 2013) but this is the first demonstration of this phenomenon in *Neisseriae*.

Results

US_NmUC isolates demonstrate heteroresistance to polymyxin B and colistin

AMP resistance is an important pathogenic trait for both Ng and Nm. PmB E-test strips were used to determine the PmB MICs and revealed that the non-encapsulated US_NmUC isolates were highly resistant (Supplemental Table S1; Figure 1A). Of 52 CNM isolates, 41 (79%) had PmB MIC of 128-256 μg/ml and MICs of 10/11 remaining isolates were 64-96 μg/ml. One isolate, CNM34, had a significantly lower PmB MIC (16 μg/ml). Whole genome analysis of this isolate revealed a 2-bp deletion in *mtrC*, which encodes a key component of the Mtr efflux pump (Shafer *et al.*, 1998). Two US_NmUC isolates from Atlanta had MICs of 96 and 128 μg/ml, respectively (Table S1). For comparison, the PmB

MIC of a well-characterized unencapsulated meningococcal M7 strain (Swartley & Stephens, 1994, Tzeng *et al.*, 2005) is 64 µg/ml. The PmB MICs of a clinical gonococcal isolate recovered during the Columbus urethritis outbreak (CNG20) and two gonococcal reference strains (FA19 and FA1090) were 48, 48 and 96 µg/ml, respectively; while the PmB MIC of a multi-drug resistant (MDR) gonococcal isolate from Japan, H041, (Ohnishi *et al.*, 2011) is 192 µg/ml. These data suggest that the non-encapsulated US_NmUC isolates display equal or greater AMP resistance than gonococci.

Many of the US_NmUC isolates displayed PmB heteroresistance (Li *et al.*, 2006, Lo-Ten-Foe *et al.*, 2007); i.e., they yielded subpopulations of highly PmB resistant colonies in the zone of inhibition in disc diffusion and E-test assays (e.g. CNM3 shown in Figure 1A). These isolates also yielded higher MIC values in microbroth dilution assays. For example, the PmB MIC of CNM3 was 256 µg/ml by E-test, but was 1,024 µg/ml using the microbroth dilution assay. In contrast, neither the gonococcal strains tested nor M7 exhibited heteroresistance. However, during E testing, we did observe heteroresistant colonies of FAM18, a serogroup C cc11 reference strain, and a serogroup W cc22 invasive isolate GA18736 from Georgia (E-test pictures shown in supplemental figure 1), indicating that heteroresistance in Nm is not only found in US_NmUC isolates.

Heteroresistance of the US_NmUC isolates was confirmed using population analysis profiling (PAP) assays (El-Halfawy & Valvano, 2015). CNM3, CNM8 and the non-encapsulated FAM18 derivative FM7 was not eliminated by > 16-fold increases in PmB concentration (Figure 1B). In contrast, growth inhibition of M7 and FA1090 occurred across a narrow PmB concentration range. MC58 and FA19 also did not exhibit heteroresistance (data not shown). Interestingly, although CNM8 and FM7 failed to form colonies in the zone

of inhibition in E tests (Figure 1A), these strains were heteroresistant in the PAP assay (Figure 1B).

We also examined whether the isolates exhibiting PmB heteroresistance would display analogous phenotypes toward another clinically used AMP, colistin (polymyxin E). As shown in Figure 1C, CNM3 and FM7 displayed the heteroresistance profile toward colistin; while the growth of M7 and FA1090 was inhibited across a narrow range. These data were consistent with the PmB resistance profiles of these strains. Further, the colistin E-test of CNM3 also showed colonies within the zone of inhibition (insert in Fig. 1C).

Heteroresistance to PmB is stable in US_NmUC isolates

Heteroresistance to PmB, colistin and other antimicrobial agents in some bacteria is transient and reverts in the absence of continuous antimicrobial pressure (Napier *et al.*, 2014, El-Halfawy & Valvano, 2015). To test if heteroresistance in the US_NmUC isolates was reversible, colonies picked from the zones of inhibition of CNM isolates (Table 1) were repeatedly passed on GCB agar plates in the absence of PmB. The elevated PmB MICs of these colonies were retained, suggesting that their enhanced PmB resistance was stable and likely due to genetic change(s). One of the recovered heteroresistant mutants, 3R4, was examined for resistance to colistin using PAP and E-test. The mutant was more resistant to colistin with a MIC greater than 256 μg/ml, whereas the MIC of the parental isolate CNM3 to colistin was ~ 48-64 μg/ml (Figure 1C). The resistance to LL-37 was also compared. The strains were treated with varying concentrations of LL-37 for 30 min followed by plating for viable CFU counts. The 3R4 mutant was more resistant to LL-37 in all concentrations tested than the parental strain (Figure 2), thus supporting the hypothesis that PmB heteroresistance confers cross-resistance to host endogenous antimicrobial peptides.

Identification of heteroresistance associated mutations using genome sequencing

Genomes of eight heteroresistant colonies (3R3, 14R2, 17R1, 32R1, 32R2, 33R1, 37R1 and 45R2) derived from 7 CNM isolates (first number in strain designation is the parental CNM number, Table 1) were sequenced. Variants were identified by aligning the raw sequence reads against a CNM10 reference genome using PATRIC (www.patricbrc.org). Separately, assembled contigs were also compared to the CNM10 genome using the genome comparator (www.pubmlst.org).

Variants (Supplemental Data set S1) were examined for genes with previously reported roles in antimicrobial resistance. Alterations in several *pil* genes were identified in multiple heteroresistant mutants, *pilU* (33R1), *pilM* (45R2), *pilQ* (14R2, 17R1) and the *pilS* cassettes (silent incomplete pilin coding fragments responsible for antigenic variation of PilE) (17R1, 32R1, 32R2, 33R1 and 37R1) (Supplemental Data set S1). The class II *pilE* gene encoded near *katA* was intact in all mutants. Multiple repeated sequence motifs and slipped strand mispairing (SSM) events were detected, but none of these were in loci with known roles in AMP resistance. Overall, point mutations in *pilM* or *pilQ* were identified in seven of the eight heteroresistant mutants; while *pilM* is disrupted by an IS element in the remaining mutant (Table 1).

Frameshift mutations were identified in *pilQ* in the 3R4, 32R1, 32R2 and 33R1 mutants. 3R4 had a deletion (T) at position 167 that resulted in a premature stop at residue 57. Five additional colonies recovered from CNM3 (3R4-3R7) in independent experiments had the same deletion. 32R1 and 32R2 were isolated from CNM32 and both had a 1-bp insertion (G) at position 2229 that extended the PilQ coding region (815 aa vs. 769 aa). 33R1 had a 1-bp deletion (A) at position 1947 that truncated PilQ from 769 to 655 residues. The insertion in 32R1 changed a G_3 to a G_4 track and the deletion in 33R1 resulted in an A_6-to-A_5 transition. Whether these two short homopolymeric tracks have an increased sequence

instability associated with the slipped strand mispairing phase variation events is not clear (Saunders *et al.*, 2000, Snyder *et al.*, 2001). The 14R2 and 17R1 mutants had C-A conversions at positions 2266 and 1465 that resulted in T to P residue changes at residue 756 and 489, respectively. Since the *pilQ* frameshift and these missense mutations yielded high levels of PmB resistance (MIC 1,024 μg/ml), these observations suggest that the missense mutations in 14R2 and 17R1 likely disrupt PilQ's function as a multimeric protein complex. Two mutants had mutations in *pilM*, the first gene in the *pilMNOPQ* operon (Carbonnelle *et al.*, 2005). 45R2 had a C-T conversion that resulted in a stop codon at residue 44; while *pilM* in 37R1 was disrupted by an IS1655 insertion at position 16. The *pilM* mutants had lower PmB MICs (256-512 μg/ml) than the *pilQ* mutants.

To further evaluate the effects of various *pilQ* and *pilM* mutations, we performed PilQ Western blots on total cellular extracts of the heteroresistant mutants and the wild type strain. As shown in Figure 3, a high molecular weight multimer band, a monomer band and several smaller bands, presumably degraded products, were detected by the PilQ monoclonal antibodies, like the previously reported pattern (Nandi *et al.*, 2015). The *pilQ* frameshift mutants (3R4 and 33R1) resulting in premature truncation eliminated both of multimer and monomer bands (lanes 2 and 8). A weak and larger monomer band was detected for 32R1 (lane 7), in which the frameshift mutation resulting in a predicted larger protein; however, no multimer band was detected in this mutant. Two mutants with *pilQ* T to P residue changes (14R2 and 17R1) yielded no multimer bands, while maintaining monomer bands at comparable intensities as the wild type strain (lanes 5 and 6). Finally, the *pilM::IS1655* mutant (37R1) and the *pilM* frameshift mutant (45R2) showed reduced levels of PilQ in both multimer and monomer forms (lanes 3 and 4), indicating a probable

polar effect on PilQ protein expression, a phenomenon also observed in gonococci (Nandi *et al.*, 2015).

Independent pilM and pilQ mutations conferred enhanced PmB resistance

We previously showed that separate *mariner* transposon mutants in *pilM* and *pilP* caused increased PmB resistance (Tzeng *et al.*, 2005). Transferring the *pilM::aphA3* mutation into CNM3 was sufficient to increase the PmB MIC and the introduction of *pilQ::aphA3* into CNM3 also conferred the higher PmB MICs observed in *pilQ* frame shift mutants. To independently confirm the effects of *pilQ* T→P point mutations, constructs with the *aphA3(Kn^R)* cassette inserted immediately downstream of the *pilQ* stop codon and carrying 14R2 or 17R1 point mutations in *pilQ* were generated. Five transformants were sequenced. All five CNM3-14R2 transformants carried the expected mutation via homologous recombination, whereas 2 of 5 CNM3-17R1 transformants contained the desired mutation. E-tests confirmed that the transformants carrying the T→P mutation have enhanced PmB resistance (Supplemental Figure 1), while PmB MICs of the transformants with a wild type *pilQ* sequence were identical to that of CNM3. Introducing these mutations in the respective parental isolate (CNM14 and CNM17) also produced the same PmB resistance results as the original mutants.

The pilM and pilQ mutations reduced transformation efficiency

Disruption of type IV pilin biogenesis apparatus is known to cause competence deficiency (Georgiadou *et al.*, 2012). Thus, we expected the PmB heteroresistant mutants to have defects in transformation efficiency. Transformation efficiencies of the mutants were examined using chromosomal DNA carrying a *tonB::Ω(Sp)* mutation. As show in Table 1, all parental isolates showed transformation efficiency in the range of 10^{-4} – 10^{-5} per μg DNA. Significant reductions (>3 logs) were observed in all PmB resistant mutants. Most of the

mutants were not transformable. The 17R1 (PilQ/T489P) mutant and the 33R1 mutant with a truncated PilQ remained transformable but with a 2-order of magnitude reduction in efficiency. Despite the differences in transformation efficiency, all *pilQ* mutations yielded similar higher levels of PmB resistance than the *pilM* mutants, both mutants were incompetent in transformation. There was no correlation between PmB resistance and transformation phenotypes.

Heteroresistance requires the Mtr efflux pump and the LptA transferase

N. meningitidis intrinsic PmB resistance is mediated by LptA-mediated lipid A modification with phosphoethanolamine, the Mtr efflux pump and the capsule (Tzeng *et al.*, 2005, Spinosa *et al.*, 2007, Jones *et al.*, 2009). To test if heteroresistance required these mechanisms, we introduced *mtrD* or *lptA* mutations in CNM3. Inactivation of *mtrD* and *lptA* reduced the PmB MIC levels to 24-32 μg/ml and 0.1 μg/ml, respectively (E-test data shown in supplemental figure 1). Neither mutants formed heteroresistant colonies in E-test and disc diffusion assays. In addition, PAP assays showed no heteroresistance toward PmB or colistin in the *mtrD* mutant (CNM3D) (Figure 4). Thus, development of heteroresistance in these strains is dependent upon intrinsic determinants of PmB resistance (Tzeng *et al.*, 2005).

The US_NmUC isolates did not have higher spontaneous mutation or slipped strand mispairing frequencies

The number of point mutations and changes in monomeric tracks we observed suggested that the heteroresistant isolates might have higher mutation rates. We examined the spontaneous mutation rate in CNM3 using rifampin plating assays. The spontaneous rifampin resistance rate of CNM3 was low (median 2.2×10^{-10}) and comparable to a low switcher strain IR2781 (median 1.7×10^{-9}) (Richardson & Stojiljkovic, 2001). For comparison,

IR2855, a *mutL* mutator strain that has a ~1000-fold higher mutation rate (median 3.7×10^{-7}) than CNM3, exhibited a PAP profile similar to CNM3 (Figure 4A). The DNA mismatch repair (MMR) pathway is an important determinant of overall mutability and phase variation frequency in Nm (Richardson & Stojiljkovic, 2001). We generated a *mutL* mutation in CNM3 (CNM3L) to test if heteroresistance was influenced by the MMR pathway. The spontaneous rifampin resistance rate in CNM3L was more than 100-fold higher compared to CNM3 but these strains had similar PAP profile (Figure 4A). Thus, the MMR system did not appear to have a major role in the PmB heteroresistance phenotype.

Interestingly, it has been reported that cationic antimicrobial peptides, LL-37 and colistin, increased iron-induced mutagenesis in *P. aeruginosa* (Limoli *et al.*, 2014, Rodriguez-Rojas *et al.*, 2015). As we have performed experiments using iron-rich GC media, it was plausible that the heteroresistant mutations were enhanced in the presence of PmB and possibly other AMPs in *N. meningitidis*. We examined whether the antimicrobial peptide affected mutagenic phenotype in the clade isolate by comparing spontaneous rifampin mutation rates of meningococci grown on standard GC plates (Fe^{+3} is supplemented at 12 μg/ml) with or without colistin at 128 or 256 μg/ml (MIC_{50} as determined by the PAP assay is ~128 μg/ml). When compared to meningococci grown in the absence of colistin, no significant increases in spontaneous rifampin mutation rates upon exposure to colistin under the iron-replete condition were observed (data not shown).

We also measured the frequency of slipped-strand mispairing using the universal rate of switching cassette (UROS) assay (Alexander *et al.*, 2004b). The UROS cassette contains a poly $(G)_8$ tract within the *Ω(Sp)* cassette of *aadA* that is in the off phase. Thus, Sp^R colonies form when *aadA* is switched into the on phase by slipped strand mispairing. The UROS cassette did not show a higher slipped strand mispairing rate in CNM3, when

compared to that of strain IR5426, (UROS cassette in the high switcher/mutator IR2855) (Alexander *et al.*, 2004b).

PmB heteroresistant mutants have reduced susceptibility to multiple antibiotics

We tested if the resistance to other antimicrobial agents was altered in the PmB heteroresistant mutant 3R4, using E-tests (Table 2) and disc diffusion (supplemental table 2) on GC agar plates. CNM3 and 3R4 had similar susceptibility to levofloxacin, meropenem and azithromycin, which is one of the two current standards of care antibiotic treatment for gonorrhea. However, 3R4 has reduced susceptibility to penicillin G, ceftriaxone, cefotaxime, streptomycin, kanamycin, chloramphenicol and tetracycline (Table 2). The resistances of 3R4 to ceftriaxone and cefotaxime were two-fold higher than CNM3 by E-test, although its MICs remained in the sensitive range (Table 2). Interestingly, CNM3 has reduced susceptibility to several antibiotics such as azithromycin, meropenem, penicillin, and cefuroxime when compared to the Ng reference strain FA19 and to a clinical isolate CNG20. The resistance of CNM3 to penicillin G, cefuroxime and azithromycin was like that of the gonococcal isolate MS11, which has elevated resistance due to the presence of the *penA*, *mtrR*, and *penB* mutations (Ropp *et al.*, 2002, Ohneck *et al.*, 2011). Thus, the *pilQ* defect in the PmB heteroresistant mutant indeed influenced, albeit modestly, the susceptibility to many unrelated antibiotics.

Altered resistances to penicillin G, chloramphenicol and tetracycline between CNM3 and 3R4 were also compared in the *mtrD* and the *lptA* mutant backgrounds and no differences were observed in these backgrounds (Table 3). These data suggest that the higher intracellular antibiotic levels caused by either efflux pump inactivation (*mtrD*) or compromised membrane integrity (*lptA*) cannot be effectively reduced by blocking antibiotic entry through a *pilQ* mutation.

Discussion

The US_NmUC has emerged as urogenital pathogen. Historically, Nm is not recognized as a significant cause of urogenital infection and the occasional meningococci recovered from such isolated cases have been from diverse serogroups and lineages (Harrison *et al.*, 2017, Ma *et al.*, 2017). The recent urethritis outbreaks and clusters caused by US_NmUC suggest that with novel genetic and phenotypic changes, this meningococcal clade is being transmitted efficiently between sexual partners and can successfully resist local innate immune responses (Bazan *et al.*, 2016, Tzeng *et al.*, 2017, Bazan *et al.*, 2017, Toh *et al.*, 2017, Retchless *et al.*, 2018).

Like *N. gonorrhoeae*, the US_NmUC isolates trigger a potent local inflammatory response characterized by urethral discharge and presence of many polymorphonuclear leukocytes (PMNs) in the inflammatory exudates (Johnson & Criss, 2011). These US_NmUC isolates exhibited bacterial resistance to host killing. In this report, we investigate the basis of high-level resistance to AMPs of these US_NmUC isolates. Resistance to host-derived AMPs is an important pathogenic trait for both Ng and Nm (Johnson & Criss, 2011). Recent studies have indicated that PMNs primarily direct non-oxidative antimicrobial activities against Ng (Johnson & Criss, 2011). Non-encapsulated Nm and Ng are generally more sensitive to the action of AMPs than encapsulated meningococci (Tzeng *et al.*, 2005). The ability of US NmUC isolates to resist killing by human AMPs, either produced locally by the mucosal epithelia where they serve as a primary defense mechanism or to resist AMP-mediated non-oxidative killing by PMNs at this site, may have important advantages. Survival in PMN's may also serve as vehicles for dissemination in urethral exudates. Many of the non-encapsulated US_NmUC isolates displayed greater PmB resistance (MICs 96-256

μg/ml) than Ng and considerable higher resistance (MICs 384-1,024 μg/ml) was observed for the heteroresistant subpopulations.

Heteroresistance to PmB or colistin has been described in *Acinetobacter baumannii* (Li *et al.*, 2006), *Enterobacter cloacae* (Lo-Ten-Foe *et al.*, 2007), *P. aeruginosa* (Hermes *et al.*, 2013), *Salmonella typhimurium* (Hjort *et al.*, 2016), *Klebsiella pneumonia* (Jayol *et al.*, 2015, Band *et al.*, 2018) and *Burkholderia cenocepacia* (El-Halfawy & Valvano, 2013) and can lead to treatment failure in clinical settings and in experimental models (Band *et al.*, 2016, Band *et al.*, 2018). Interestingly, the PmB heteroresistant subpopulation of *B. cenocepacia* can protect the more sensitive *B. cenocepacia* population as well as sensitive *P. aeruginosa* and *Escherichia coli* from killing by PmB and various bactericidal antibiotics (El-Halfawy & Valvano, 2013). However, heteroresistance to AMPs has not been previously reported for *Neisseria*. Further, we found that meningococcal heteroresistance was not transiently induced upon exposure to AMPs (Napier *et al.*, 2014), but was instead stable in the absence of PmB, and we have identified the genetic alteration(s) aiding PmB resistance.

Mutations in the pilin biogenesis apparatus appear to restrict the entry of PmB and other antimicrobial agents, and responsible for heteroresistance in Nm. PilQ secretin, in addition to promoting pilin biogenesis and DNA transformation, facilitates the entry of small molecules into the bacterial cell. High-level heteroresistance to PmB can produce cross-resistance to several other important antimicrobial agents [Table 2 and (Napier *et al.*, 2014)]. The increase in MICs of PmB and to a variety of antibiotics in the heteroresistant mutants compared to the wild type strain are consistent with those observed in gonococcal *pilQ* mutants (Chen *et al.*, 2004, Zhao *et al.*, 2005, Johnson *et al.*, 2014), highlighting the importance of a functional PilQ in the entry of antibiotics.

PilQ facilitates the entry of a variety of antibiotics in the gonococci, including penicillin, ceftriaxone, vancomycin, tetracycline, rifampin, and ciprofloxacin (Ropp *et al.*, 2002, Zhao *et al.*, 2005, Nandi *et al.*, 2015). An *in vitro* spontaneous mutation screen of two gonococcal isolates containing mosaic *penA* sequence with MICs to ceftriaxone ranging from 0.03 to 0.06 µg/ml identified mutants with increased MICs to ceftriaxone almost 10-fold (0.5 µg/ml). Genetic analysis showed an identical 2-bp insertion in *pilQ* in each of the mutants (Johnson *et al.*, 2014). Further, spontaneous penicillin resistant gonococcal clones were selected at a frequency of ~10^{-6} and all had a non-piliated morphology (Nandi *et al.*, 2015). The mutations were found to be clustered within the C-terminal domain (residue 400 to 731) of PilQ and all *pilQ* mutants increased the MIC of penicillin by 2.5- to 3-fold (Nandi *et al.*, 2015).

Of the five gene mutations known to contribute to high-level penicillin resistance in Ng (Ropp *et al.*, 2002), *penB*, *mtrR* and *pilQ2*, have been shown to also play a role in PmB susceptibility (Tzeng *et al.*, 2005). The *pilQ2* allele is an E666K point mutation in *pilQ* (Zhao *et al.*, 2005). Interestingly, the increased resistance due to acquisition of the *pilQ2* mutation is observed only in strains containing the *mtrR* and *penB* resistance determinants (Zhao *et al.*, 2005). The diffusion of antibiotics through PilQ become significant only when influx through porins is limited due to disruptions in the porin gene (*penB*) or up-regulation of Mtr pump efflux (*mtrR*), (Zhao *et al.*, 2005). Similarly, diffusion of antibiotics through PilQ is likely only a small fraction of the antibiotic influx in *N. meningitidis*, as reflected by the modest changes in MIC values in the heteroresistant mutants. We observed that the *pilQ* mutations caused enhanced PmB resistance only in the wild type background. When resistance levels

were reduced ~4-8 fold by mutations in the Mtr efflux pump, we did not observe any clear difference in PmB resistance with the combined *pilQ/mtr* mutation.

Defects in the mismatch repair (MMR) pathway responsible for removing insertion/deletion loops (Lahue *et al.*, 1989) have generally been associated with meningococcal mutator phenotype (Richardson & Stojiljkovic, 2001, Richardson *et al.*, 2002). Since PmB resistant mutants due to frameshift and point mutations in *pilQ* and *pilM* were recovered readily from the heteroresistant clade isolates, a possible mutator phenotype and a defect in MMR pathway was explored. Nevertheless, the clade isolates did not show enhanced mutation rate using the standard spontaneous rifampin resistance plating assay and have relatively low slipped strand mispairing rates. We examined the contribution of the MMR pathway by introducing a *mutL* mutation, and confirmed that no effect on heteroresistance was detected in the *mutL* mutant. However, the standard rifampin plating assay that measures the spontaneous mutation rate in the essential *rpoB* gene, encoding a subunit of RNA polymerase, is likely constrained to only detect the rate yielding viable mutants. Thus, it is possible that the mutation rates obtained with rifampin resistance do not reflect the rates of those mutations needed for antimicrobial peptide resistance. Antimicrobial peptides such as colistin and LL-37 have been reported to enhance mutation rates in *P. aeruginosa* (Limoli *et al.*, 2014, Rodriguez-Rojas *et al.*, 2015). However, we did not detect significant changes in rifampin mutation rates when meningococci were grown on iron-rich GC agar plates in the presence of colistin, a condition shown to influence mutagenic phenotype in *P. aeruginosa* (Rodriguez-Rojas *et al.*, 2015).

Other repair pathways correcting DNA lesions include the base excision repair (BER) (MutY, Fpg/MutM, Nth), the nucleotide excision repair (NER) (UvrA/B/C), the recombinational repair (RecA/B/C/D), and translesion synthesis (DinB) (Davidsen *et al.*,

2007b). Several of these DNA repair proteins and others have been characterized to influence mutation rates (Alexander *et al.*, 2004a, Martin *et al.*, 2004, Davidsen *et al.*, 2007b, Davidsen *et al.*, 2007a), thus additional studies are needed to have a detailed understanding about the contribution of other DNA repair pathways in the development of heteroresistance.

The 37R1 heteroresistant mutant had a *pilM* disruption by a newly inserted IS*1655*. IS*1655* is a 1080-bp long element that would generate a 3-bp target duplication upon insertion (Kiss *et al.*, 2007), which was indeed observed in 37R1. A whole genome comparison study of disease and carriage strains has suggested that Nm can be separated from *Neisseria lactamica* and Ng based on the respective IS repertoires and that IS*1655* is restricted to Nm (Schoen *et al.*, 2008). The authors noted that none of the six Nm strains analyzed have IS*1655* at the same chromosomal location, suggesting a high mobility of IS*1655*. Inspecting the complete genome of the US_NmUC isolate CNM10, we found 11 intact copies and one truncated copy of IS*1655*. As a comparison, the serogroup B strain MC58 has 14 copies; while the cc11 reference FAM18 has 9 intact and 1 truncated copies. Since the genome of 37R1 had multiple contig breaks, it is uncertain whether 37R1 has the same copy number of IS*1655* as CNM10.

Because the pilin biogenesis mutations disrupt normal piliation, the increases in AMP resistance because of such mutations might not be biologically significant, considering the importance of pili in colonization and infections. However, mutations in *pilQ* have been shown to result in complex phenotypes (Helm *et al.*, 2007), it is possible that certain *pilQ* mutations allow for increased AMP resistance and retain pathogenic potential. Interestingly, experimental infections of male volunteers using a nonpiliated gonococcal *pilE* mutant

showed that the pilus was not required for infection, although the symptoms were less severe in infections with the nonpiliated variant (Hobbs *et al.*, 2011).

Ng has been proposed to have originated from Nm, a pharyngeal colonizer that switched to primarily colonizing the urogenital tracts, resulting in lower frequency of gene flow between Nm and Ng due to ecological separation within the human host (Vazquez *et al.*, 1993). The ability of US_NmUC isolates to withstand killing by AMPs produced either by epithelial cells or by PMNs is likely an important selective advantage and requirement for dissemination in urethral exudates. Further, the ability of the clade isolates to effectively colonize the urogenital tract raises the concern of horizontal gene transfer of antimicrobial resistance determinants, considering the wide-spread antimicrobial resistance in Ng (Unemo & Shafer, 2014). US_NmUC isolates are intermediate in sensitivity to penicillin, sensitive to azithromycin and ceftriaxone and have uniformly responded to gonococcal treatment regimens. However, the heteroresistance phenotype further demonstrates the propensity for enhanced antibiotic resistance by spontaneous mutations of the pilin biogenesis genes and/or IS movement in this clade. Based on emergence of antibiotic resistance in the gonococcus, we will continue to need to monitor the antimicrobial resistance of this novel meningococcal urethritis clade.

Experimental Procedures

Bacterial isolates and growth conditions

Bacterial strains used in this study are listed in Table 4. These stains included 52 *N. meningitidis* urethritis clade isolates collected from men between January 2015 and September 2015 at Columbus Public Health (CPH), Columbus, Ohio (Tzeng *et al.*, 2017) with

the CNM3 isolate being the major representative of this collection. The initial demographic features of these cases and the collection protocol have been previously reported (Bazan *et al.*, 2016, Bazan *et al.*, 2017). Two additional clade isolates from Atlanta, Georgia, ATL#1 and ATL#2, a serogroup A isolate, IR2855, a serogroup W clinical isolate, GA18736, as well as genetically defined derivatives of well-characterized *N. meningitidis* strains IR2781 (NMB) (Stephens *et al.*, 1991) and FAM18 were also used. One gonococcal isolate (CNG20) recovered from the same period and gonococcal reference strains, FA19, FA1090, MS11 and H041 were also used for comparisons. *Neisseria* were cultured with 5% CO_2 at 37°C on GC base (GCB; Difco) agar containing supplements of 0.4% glucose and 0.68 mM $Fe(NO_3)_3$, or GC broth with the same supplements and 0.043% $NaHCO_3$. Brain heart infusion (BHI) medium with 1.25% fetal bovine serum was used when kanamycin selection was required. *Escherichia coli* strains were routinely grown in Luria Bertani broth for cloning and propagation of plasmids. *N. meningitidis* was transformed by the procedure of Janik et al. (Janik *et al.*, 1976). *E. coli* strains were transformed by chemical competence or by electroporation with a GenePulser (Bio-Rad) according to the manufacturer's protocol. When necessary, *Neisseria* (*E. coli*) were grown in the presence of antibiotic concentrations (μg/ml): kanamycin (Kn) 80 (50), chloramphenicol, 5 (34), tetracycline, 5 and spectinomycin (Sp), 60 (100).

Susceptibility assays

The minimum inhibitory concentrations of PmB and antibiotics were determined by E test. Cell suspensions from overnight GC plates adjusted to OD_{550} of 0.3 were swabbed onto GC agar plates. Discs soaked with 10 μl of PmB solutions (25.6 mg/ml) or discs with defined levels of antibiotics (BBL) were overlaid and the plates were incubated overnight at

37°C in 5% CO_2. E test strips (bioMerieux) were performed in a similar fashion. MIC values of PmB were reported as µg/ml. Microbroth dilution assays using 96-well microtiter plates were performed using GC broth with standard supplements. Two-fold serial dilutions of PmB concentrations starting at 1,024 µg/mL were tested. The sensitivity to LL-37 was determined using 96-well microtiter plate.

Population analysis profiling (PAP) assays

Overnight plate cultures of meningococcal strains were resuspended in GC broth and adjusted to optical density of 0.3 at 550 nm. Ten-fold serial diluted bacterial suspensions were prepared and duplicate aliquots of 40 µl of suspensions were spotted onto GC agar plates with 2-fold incremented concentrations of polymyxin B. Bacterial growth at each of these concentrations is quantified by CFU count after overnight incubation. An isolate would be considered heteroresistant when the lowest antibiotic concentration giving maximum growth inhibition is >8-fold higher than the highest non-inhibitory concentration (El-Halfawy & Valvano, 2015).

Western blot

Expression of PilQ in whole-cell extracts was examined by Western blot. Briefly, strains grown on GC plates overnight at 37°C were collected by centrifugation. Whole cell lysates of equal cell densities were prepared in SDS loading buffer, resolved by 8% SDS-PAGE, and transferred to nitrocellulose membranes by semi-dry transfer. The antisera against PilQ (Tonjum et al., 1998) were used at 1:5000 dilutions and anti-rabbit IgG-HRP conjugate secondary antibody (Bio-Rad) was used at 1:10,000 dilution. The blot was developed using a 1:5 dilution of pico chemiluminescent substrate (Pierce).

LL-37 killing assay

Overnight cultures were harvested into RPMI and adjusted to OD_{550} of 0.3. The standardized suspensions were diluted 100-fold and then 50-fold to have approximately 10^5 CFU/ml. Each assay was started by the addition of 90 µl of cells into a well containing 10 µl of LL-37 to reach the desired final concentrations, 1.25 to 10 µg/ml. The microtiter plate was incubated at 37°C and 5% CO2. Two 20-µl aliquots of the sample were removed after 30 min and the number of viable CFU were determined by plating onto GC agar plates. Experiments were performed in duplicate wells on several occasions. Student's t test was used to determine the statistical significance of survival of the mutant with respect to that of the wild type strain, with P values of < 0.05 considered significant.

Whole genome sequencing (WGS) and variant analysis

WGS of all 52 CNM isolates has been performed by Illumina at CDC (Tzeng *et al.*, 2017). The single contig genome of isolate CNM10 sequenced by Pacific Biosciences (PacBio) technology (Tzeng *et al.*, 2017) was used as the reference genome in variant analysis. Polymyxin B resistant derivatives within the zone of inhibitions were recovered from seven CNM urethritis isolates (Table 1) were sequenced by MiSeq, yielding ~200X coverage of paired end 250-bp reads, assembled using SPAdes (Bankevich *et al.*, 2012) and annotated by RAST(Aziz *et al.*, 2008). The variant analysis service provided at www.patricbrc.org was utilized with the BWA-men (Li & Durbin, 2009) and SAMtools (Li *et al.*, 2009) programs as the aligner and the SNP caller, respectively. The Illumina raw reads of the mutants were analyzed against a complete CNM10 genome as the reference. In addition, the assembled contigs were compared to the reference CNM10 genome using the genome comparator tool

available at the PUBMLST site (www.pubmlst.org) to identify allele differences. Variants were further confirmed by targeted PCR amplification and sequencing.

Construction of lptA, mtrD/E, pilM, pilQ,* and *mutL mutants

The CNM3 isolate was transformed with pKA314 (Tzeng *et al.*, 2004) to generate the *lptA::Ω(Sp)* mutant, CNM3A. The *mtrE:: Ω(Kn)* mutation was PCR amplified from the M7mtrE mutant with primers mrtDF2-ER and mtrE3R1-ER and the *mtrD:: Ω(Sp)* mutation from strain XZ134 (Tzeng *et al.*, 2005) using primers mtrCF1 and mtrE3R1-ER. The purified PCR products were then used to transform CNM3. The *lptA*/*mtrE* double mutant, CNM3EA, was subsequently generated by transforming the *mtrE* mutant with pKA314. A PCR product with the *mutL::Ω(Sp)* mutation was obtained with primers mutL1a and mutL1b (Richardson & Stojiljkovic, 2001) and used to transform the CNM3 isolate.

The *pilM::aphA3* mutation was created by the overlapping PCR method. Primer pairs of YT113 and pilM-5Ra and primer pairs of pilM-3Fa and pilMR2 were used to generate 5'627-bp and 3'460-bp fragments, respectively. The *aphA3(Kn)* cassette was amplified using primers aphA3-SmF and aphA3-SmR. First overlapping PCR was performed with the *aphA3* cassette and the 3' fragment and then the resulting product was used for the second overlapping with the 5' fragment. The resulting construct deleted 756-bp *pilM* sequence.

To introduce the *pilQ* point mutations found in the PmB heteroresistant mutants 14R2 and 17R1, a construct with *aphA3* cassette inserted 25 bp downstream of the *pilQ* stop codon was created by overlapping PCR. Primers pilQ-F1 and pilQ-5RA3 were used to amplify an 1144-bp 5' fragment from chromosomal DNAs of isolate 14R2 and 17R1 that carries the respective point mutations. The first overlapping PCR combined the 5' fragment with the *aphA3* cassette and the resulting product was subsequently used for overlapping PCR with a

3' 849-bp fragment of primers pilQ-3FA3 and pilQ-3R2. The final ~2.8 kb product was used to transform either the CNM14 or CNM17 parental isolates as well as the CNM3 isolate and kanamycin resistant colonies were saved. PCR products were generated from the colonies and sequenced to determine whether the point mutations were recombined into the transformants. Transformants with the desired point mutations or with a wild type sequence were saved for comparison. The CNM3DQ and CNM3AQ mutants were created by transformation of strains CNM3D and CNM3A with the overlapping PCR products carrying the 14R2 *pilQ* mutation.

Determination of spontaneous mutation and slipped strand mispairing frequencies

Overnight GC plate cultures were resuspended in GC broth and standardized by the OD_{550} readings. For spontaneous mutation rates, 40 µl of serial dilutions of cell suspensions were spotted onto GC plates for total CFU counts. Approximately 10^{10} cells were plated onto GC plates containing 3 µg rifampin/ml (Richardson & Stojiljkovic, 2001). Spontaneous rifampin mutation rates were obtained as the ratio of rifampin-resistant cells to the total number of cells. Serial dilutions of UROS-containing strains were plated on non-selective GC plates for total counts and on selective (60 µg/ml spectinomycin) plates for switch-on CFU counts. Frequencies of phase variation were determined as described previously (Alexander *et al.*, 2004b) and are represented as medians of three independent measurements.

Transformation efficiency

Plate-grown meningococcal strains were suspended in GC broth supplemented with 5 mM $MgCl_2$. One µg of chromosomal DNA (*tonB::ΩSp*) from Nm strain NMB was added to aliquots (100 µl) of cell suspension at an OD_{550} of 1 and then incubated for 1 hr at 37°C. Pre-warmed GC broth with complete supplements (500 µl) and DNase I (2 units) was added and

the incubation continued for another 30 minutes. Serial dilutions were made and aliquots of 50 µl were spotted onto non-selective GC plates and the colony forming units (cfu) determined after overnight growth. 500-µl of the transformation mixtures and 100-µl aliquots of 10^{-1} and 10^{-2} dilutions of the mutants and the parental strains, respectively, were plated onto selective (Sp) plates. The efficiencies were calculated as the ratio of cfu/ml from the selection plate to the cfu/ml of non-selective plates.

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The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Author Contributions

YT and DSS designed the research and wrote the manuscript. ET, DEN, ACR and XW contributed to WGS analyses. YT, ET, ZB, DEN, XW, ACR, JAB, ANT contributed to the acquisition, analysis, or interpretation of the isolates and of the data. All authors read and approved the final submitted version of the manuscript.

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Table 1. Polymyxin resistant mutants derived from the US_NmUC isolates

Mutant	**PmB MIC (parental)**	**Gene**	**Position**	**Changes[a] Parent/mutant**	**Outcome[b]**	**Transformation frequency[c] (parental)**
3R3, 3R4, 3R5, 3R6, 3R7	1,024 (128)	*pilQ*	T167	ctTcg/ctcg	fs F56S*	$<1.3\times10^{-8}$ (3.4×10^{-4})
14R2	1,024 (192)	*pilQ*	A2266	acc/Ccc	T756P	$<4.9\times10^{-9}$ (1.9×10^{-5})
17R1	1,024 (128)	*pilQ*	A1465	acc/Ccc	T489P	**3.2×10^{-7}** (2.1×10^{-5})
32R1, 32R2	1,024 (128)	*pilQ*	G2229	ggg/gggG	fs G743 → 815 aa	$<1.4\times10^{-8}$ (4.1×10^{-5})
33R1	1,024 (96)	*pilQ*	A1947	Aaaaaa/aaaaa	fs AVLG/PSWG 655*	**1.3×10^{-7}** (2.2×10^{-5})
37R1	384 (96)	*pilM*	A16	IS1655 insertion	Disruption	$<3.9\times10^{-8}$ (3.6×10^{-5})
45R2	384 (128)	*pilM*	C126	caa/Taa	fs Q43*	$<7.2\times10^{-9}$ (7.9×10^{-5})

a: For point mutation, the wildtype sequence is shown on the left and the mutant on the left. The changed nucleotide was in capital letter.

b: Amino acid changes from the wild type to the mutant and the residue number are indicated. An asterisk indicates a stop codon immediately following the residue. The frameshift in 32R1 and 32R2 removed the original stop codon and yielded a larger PilQ protein. The lengths of PilQ and PilM proteins are 769 and 371, respectively.

c: Transformation was performed using 1 μg chromosomal DNA carrying a *tonB::Ω(Sp)* mutation. Frequencies (n=3) were calculated as the ratio of Sp^R cfu/ml to total cfu/ml per 1 μg DNA. The mutants with numbers in bold were transformable at low frequencies; while no transformants were recovered from the others.

Table 2. Comparison of antibiotic resistance levels of the PmB heteroresistant mutant[#]

Antibiotics	CNM3	CNM3R4	MS11	CNG20	FA19
	E test (μg/ml)				
Colistin	**53.3 ± 9.2**	**256****	256	48	32
Penicillin G	**0.22 ± 0.08**	**0.46 ± 0.10****	0.25	0.064	0.012
ceftriaxone	**0.002 ± 0.000**	**0.004 ± 0.001****	0.002	0.002	0.002
Cefotaxime	**0.01 ± 0.003**	**0.021 ± 0.004****	0.012	0.004	0.002
Azithromycin	0.70 ± 011	0.85 ± 0.14	0.19	0.032	0.064
Streptomycin	**7.8 ± 0.7**	**10.9 ± 2.0****	1024	4	6
Kanamycin	**10.9 ± 2.0**	**14.3 ± 2.1****	8	8	4
Chloramphenicol	**0.63 ± 0.13**	**0.81 ± 0.18***	2	0.125	0.25
Meropenem	0.3 ± 0.1	0.3 ± 0.1	0.064	0.094	0.064
Tetracycline	**0.19 ± 0.0**	**0.26 ± 0.1***	0.5	0.25	0.094
Levofloxacin	0.008 ± 0.0	0.007 ± 0.0	0.004	0.002	0.003

#. Data are presented as the mean ± standard deviation (N=3-8). The MIC values of antibiotics in bold are statistically different between CNM3 and 3R4 by student's t test (**, $p < 0.01$; *, $p < 0.05$). A single data point is shown for each gonococcal strain as comparison.

Table 3. Comparison of antibiotic resistance levels of the PmB heteroresistant derivative in the *mtrD* or *lptA* mutant background

Antibiotics	CNM3D	CNM3DQ	CNM3A	CNM3AQ
	E test (μg/ml)			
Penicillin G	0.053 ± 0.01	0.058 ± 0.01	0.079 ± 0.021	0.115 ± 0.018
Chloramphenicol	0.38 ± 0.0	0.38 ± 0.0	0.38 ± 0.0	0.38 ± 0.0
Tetracycline	0.182 ± 0.151	0.115 ± 0.018	0.094 ± 0.0	0.084 ± 0.017

#. Data are presented as the mean ± standard deviation (n=3).

Table 4. Bacterial strains used in this study.

Strains	Description	Source
IR2781	*N. meningitidis* serogroup B strain NMB	(Richardson & Stojiljkovic, 2001)
FM7	Non-encapsulated *N. meningitidis* serogroup C strain FAM18	This study
M7	Non-encapsulated serogroup B strain IR2781	(Swartley & Stephens, 1994)
GA18736	*N. meningitidis* serogroup W 2002 clinical isolate	Laboratory collection
IR2855	*N. meningitidis* serogroup A clinical isolate	(Richardson & Stojiljkovic, 2001)
IR5426	*hpuB::UROS* derivative of IR2855	(Alexander *et al.*, 2004b)
CNM3	*N. meningitidis* US_NmUC isolate	(Tzeng *et al.*, 2017)
CNM8	*N. meningitidis* US_NmUC isolate	(Tzeng *et al.*, 2017)
CNM3uros	*hpuB::UROS* derivative of CNM3	This study
3R4	*N. meningitidis* PmB heteroresistant derivative of CNM3	This study
14R2	*N. meningitidis* PmB heteroresistant derivative of CNM14	This study
17R1	*N. meningitidis* PmB heteroresistant derivative of CNM17	This study
32R1	*N. meningitidis* PmB heteroresistant derivative of CNM32	This study
32R2	*N. meningitidis* PmB heteroresistant derivative of CNM32	This study
33R1	*N. meningitidis* PmB heteroresistant derivative of CNM33	This study
37R1	*N. meningitidis* PmB heteroresistant derivative of CNM37	This study
45R2	*N. meningitidis* PmB heteroresistant derivative of CNM45	This study
CNM3-14R2	*N. meningitidis pilQ* mutation of 14R2 incorporated into CNM3	This study
CNM3-17R1	*N. meningitidis pilQ* mutation of 17R1 incorporated into CNM3	This study
CNM3L	*N. meningitidis* CNM3/*mutL::aphA3*	This study
CNM3E	*N. meningitidis* CNM3 with *mtrE:: Ω(Kn)* mutation	This study

CNM3D	*N. meningitidis* CNM3 with *mtrD::Ω(Sp)* mutation	This study
CNM3A	*N. meningitidis* CNM3 with *lptA:: Ω(Sp)* mutation	This study
CNM3EM	*N. meningitidis* CNM3E with *pilM:: aphA3* mutation	This study
CNM3DQ	*N. meningitidis* with the *pilQ* mutation of 14R2 incorporated into CNM3D	This study
CNM3AQ	*N. meningitidis* with the *pilQ* mutation of 14R2 incorporated into CNM3A	This study
FA1090	*N. gonorrhoeae* reference strain	Laboratory collection
FA19	*N. gonorrhoeae* reference strain	Laboratory collection
CNG20	*N. gonorrhoeae* urethritis isolate	(Tzeng *et al.*, 2017)
H041	MDR *N. gonorrhoeae* clinical isolate	(Ohnishi *et al.*, 2011)

Figure legends

Figure 1. (A) E-test pictures of two US_NmUC isolates (CNM3 and CNM8), two non-encapsulated Nm reference strains (M7 and FM7) and Ng reference strains GA1090. The arrow points to a heteroresistant colony in the zone of growth inhibition. **(B)** Population analysis profiling (PAP) assays of PmB with strains M7 (□), CNM3 (■), CNM8 (○), FM7 (◆) and FA1090 (●). The CFU counts of plates without PmB were set as 100% for normalization. The dotted line indicates the limit of detection. Each PmB concentration was assayed in triplicate and the experiments were repeated at least three times. **(C)** PAP assays of colistin performed similarly to PmB PAP assays with strains CNM3 (■), PmB heteroresistant mutant 3R4 (○), FM7 (◆), M7 (□) and FA1090 (●). Each concentration was tested in duplicates and the assays repeated twice.

Figure 2. Sensitivity to LL-37 of the CNM3 clade isolate (gray) and its heteroresistant mutant 3R4 (black). Bacterial cells were incubated with LL-37 in RPMI at the indicated concentrations for 30 min and the number of viable CFU were determined by plating onto GC agar plates. Each conditions were assayed in duplicate at least twice. The averages and standard deviations of two independent assays are presented. Student's *t* test was used to determine the statistical significance of survival of the mutant with respect to that of the wild type strain (**, $P < 0.01$).

Figure 3. PilQ expression determined by Western blots. Equal amounts of whole cell lysates were resolved on 8% SDS-PAGE gels and transferred to PVDF membranes. The membrane was probed with PilQ antisera (Tonjum *et al.*, 1998). Lanes: 1, WT; 2, 3R4 (*pilQ*-frameshift); 3, 37R1 (*pilM::IS1655*); 4, 45R2 (*pilM*-frameshift); 5, 14R2 (*pilQ*/T756P); 6, 17R1

(*pilQ*/T489P); 7, 32R1 (*pilQ*-frameshift); 8, 33R1 (*pilQ*-frameshift); 9, WT. The locations of PilQ multimer and monomer were marked on the right. Protein MW ladders were labeled on the left.

Figure 4. (A) Population analysis profiling (PAP) assays of PmB resistance. Strains CNM3 (■), its *mutL* (CNM3L, ◆) and *mtrD* (CNM3D, ●) mutants and the mutator strain IR2855 (○) were compared. The CFU counts of plates without PmB were set as 100% for normalization. Each PmB concentration was assayed in triplicate and the experiments were repeated at least three times. The dotted line indicates the limit of detection. **(B)** PAP assays of colistin resistance. Strains CNM3 (■), its *mutL* (CNM3L, ◆), *mtrD* (CNM3D, ●) and heteroresistant mutant 3R4 (○) were examined analogously to the PmB PAP assays.